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JAWAHARLAL NEHRU UNIVERSITY
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SCHOOL OF LIFE SCIENCES

Prof. P.C. Kesavan

August 19, 1987

Dear Dr. Iya,

I am attaching herewith a copy of the report on the question of induction of polyploidy cells and chromosomal aberrations in experimental animals and children-fed irradiated wheat.

As you might be aware, the Ministry of Health, Government of India, had set up a Committee consisting of Prof. P.V. Sukhatme and myself to go into the entire question of the reported study, especially the irradiated wheat by the National Institute of Nutrition, Hyderabad under Dr. Gopalan and of the Bhabha Atomic Research Centre. We made a thorough study of all the available data. We also examined number of slides with microscopes and we have come to the conclusion that the irradiated wheat (75 Krad) does not induce polyploidy cells and other chromosomal aberrations. The report which I am sending has been authenticated by my initials on every page. I have no objection this scientific report being circulated to any one India or elsewhere for scientific purposes.

We had submitted this report to the Secretary, Ministry of Health, Government of India, around July 18, 1976.

With kind regards,

Yours sincerely,

P.C. Kesavan
P.C. KESAVAN

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Report on the examination of the results obtained by
NATIONAL INSTITUTE OF NUTRITION (NIN), Hyderabad and
BHABHA ATOMIC RESEARCH CENTRE (BARC), Bombay of their
studies on the Effects of Freshly Irradiated wheat on
i) lymphocytes in vitro from malnourished children;
ii) the cytology of bone marrow of rats and mice;
iii) meiotic chromosomes in male mice;
iv) germ cell survival in male mice and rats; and
v) dominant lethal mutations in rats and mice.

INTRODUCTION

In accordance with the terms of references in letter No.F.17-45/PH(F&N) of Bharat Sarkar, Ministry of Health and Family Planning, dated October 9, 1975, we, Professor P.V.SUKHATME of the Maharashtra Association for Cultivation of Sciences, Poona and Dr. P.C.KESAVAN of the School of Life Sciences of the Jawaharlal Nehru University, New Delhi, have critically examined the detailed techniques of the experiments, the appropriateness of the design of these experiments as well as the data collected and the interpretations thereof.

The Report, which we are presenting below, is entirely based on (i) the data made available to us by the Ministry along with the letter quoted above and (ii) the data published by the NIN and BARC in various national and international journals as well as their annual reports. Unpublished data, even when volunteered by either laboratories, was not considered by us, as our term of reference have been to try to identify the cause

of conflict between the data already reported for consideration by the SWAMINATHAN Committee.

The report deals with the following aspects in the following order:

1. Effects on lymphocytes in vitro from mal-nourished children fed freshly irradiated wheat.
2. Effects on the cytology of bone-marrow of rats and mice.
3. Effects of freshly irradiated wheat on meiotic chromosomes in male mice.
4. Effects of irradiated wheat on germ cell survival in male mice and rats.
5. Dominant lethal studies in rats and mice.

Of these various studies only the following experiments were independently carried out by both NIN and BARC:

1. Effect of irradiated wheat in the bone-marrow of Wistar rats;
2. Effect of irradiated wheat on the frequency of dominant lethal mutations in rats.

As suggested by the Swaminathan Committee, BARC and NIN carried out a joint study of the incidence of polyploid cells in the bone marrow of control rats. All the other experiments were carried out only by the NIN.

We have read the comments of the two Directors and also critically discussed the various points with them. The questions raised and the conclusions arrived at here are entirely/^{based} on our own analysis of the data,

1. Effects on lymphocytes *in vitro* from mal-nourished children fed freshly irradiated wheat.

The details of the study are furnished in section 1.5.2 (pages 18-20 which also includes Table 12 and in section 1.5.3.2 pages 21 and 23) of the Annual Report of NIN 1974. These data have also been published (C. Bhaskaran and G. Sadasivan, "Effects of feeding irradiated wheat to malnourished children", in the American Journal of Clinical Nutrition, 28 (1975), 130-135).

Fifteen children suffering from Kwashiorkor were divided into three groups of 5 each and fed diets containing either unirradiated, freshly irradiated or stored irradiated wheat. All the children were hospitalised for a period of 6 weeks and lymphocyte cultures were done initially and at intervals of 2 weeks. Thus, as has been pointed out by the Director, NIN (see section 1 of enclosure III, page 22 of the material provided by the Ministry) each child was its own control. The NIN data on the incidence of polyploid cells in children suffering from Kwashiorkor treated with unirradiated and irradiated wheat diets are presented in Table 12 page 20, Annual Report NIN 1974 and Table 3 of the paper published in American J. of Clinical Nutr. 28, 130-135 (1975)

The data show that children fed unirradiated wheat do not show any polyploid cells in their lymphocytes cultured by a modified technique of Arakaki and Sparkes (Cytogenetics 2, 27, 1962). However, the children fed freshly irradiated wheat had a high incidence of polyploid cells.

cells. These polyploid cells first appeared 4 weeks after the diets were started, the mean incidence at this time being 0.8%. At 6 weeks, it had increased to 1.8%. In children who had received the stored irradiated wheat, the incidence of definite polyploid cells was 0% at 4 week and only 0.6% at 6 weeks - figures considerably lower than those seen in children fed freshly irradiated wheat.

The NIN data reveal that in none of the 15 children studied, there was a single polyploid cell at the time of admission. In other words, what they had claimed was that no polyploid cell occurred in the 100 consecutive cells studied for each child. This seemed very difficult for us to accept as the occurrence of polyploid cells upto 5% in human lymphocyte cultures is well documented. In this connection we refer to table 1 on page 306 of an article entitled "A study of chromosomes of lymphocytes from patients treated with hycanthone" by G. Frota-Pessoa et al, in the Journal of Toxicology and Environmental Health, Vol.1, pages 305-307, 1975. In this paper the authors have studied 13 patients both before and after treatment with hycanthone. Hence, in this case also, each patient served as his/her own control. The data based on 100 metaphase cells before (Control) and after treatment for each individual show that the numerical variation before treatment (i.e. control) ranged from 0/100 to 5/100, (2/100 and 4/100 being more frequent). The total number of polyploids obtained in their study was 32/1300

Under these circumstances it was necessary to look into the original records maintained by the NIN and also discuss with both the authors (C. Bhaskaram and G. Sadasivan) about the discrepancy between their values and those reported in the literature. It was during this discussion on 24.6.1976 that Dr. Sadasivan deposed before the Committee that the chromosomes of these children had 'fuzzy' appearance and therefore counting the chromosomes was almost impossible. In fact he was surprised as to how his co-author (Dr. Bhaskaram) could have come to the conclusion that there were no polyploid cells in the children before their being fed on irradiated wheat.

Careful scrutinisation of the raw data revealed that the NIN value of 0.0% polyploid cells for children prior to their feeding on irradiated wheat came from only about 20 to 30 cells per child since about 75% of the cells from these malnourished children had chromosomes which exhibited "fuzzy" appearance and therefore were not fit for counting. When asked about it, Dr. Bhaskaram had no satisfactory reply to this Committee.

Secondly, the NIN has reported in the above said paper that in two children who were followed up after withdrawal of the irradiated wheat diet, the number of polyploids and abnormal cells had considerably decreased, at the end of 16 weeks and by the 24th week all the polyploid and abnormal cells had completely disappeared.

This observation is very surprising in view of the fact that the thymic lymphocytes which alone respond to

Phytohaemagglutinin (PHA) has a very long life span of the order of years and therefore it is only expected that any abnormalities induced in these cells would last for several years. This Committee asked the authors to comment on their observations which are contrary to what is expected in accordance with the biological principles. Dr. Bhaskaram had nothing to say while Prof. Sadasivan once again deposed that the entire data were imprecise and no importance should be attached thereto. The remarks of Prof. Sadasivan are extremely important not only because he is a co-author but also because he is the senior author.

Furthermore, the maximum frequency of polyploid cells observed in children fed irradiated wheat is, according to the NIN, 1.8% and this frequency is well within the normal range of incidence of polyploid cells in normal healthy human beings. This Committee therefore records that the conclusions arrived at by the NIN are not sustained.

2. Effects on the cytology of bone-marrow of rats and mice.

The minutes of the first meeting of the Scientists' Committee constituted under the Chairmanship of Dr. M.S. Swaminathan, FRS, for examining the data relating to the wholesomeness of irradiated potato, onion and wheat held on July 20, 1974 (see pages 41-58 of the material furnished by the NIN) provide as follows the data available on

(see table 1, pages 48-52), (b) comments on the data of BARC and NIN (pages 53-54) and (c) the references from the literature to the effect that megakaryocytes which form blood platelets become polyploids through endomitosis (page 54). We have come across quite a bit of literature on the occurrence of polyploid cells in the bone marrow of most mammals. Some of these are as follows:

- (1) R. Rothlin and E. Undritz (1946) (Translation from German) The megakaryocyte formation by polyploidism. Arch. Klaus. Stift. Vererb. Forsch. 21, 283-287.
- (2) E. Grundmann (1967) Studies with autoradiography and Cytophotometry in the rat liver following partial hepatectomy, In. Control of Cellular growth in adult organisms (edited by H. Tier and T. Rytomaa) Academic Press, pages 250-259.
- (3) B. G. Firkin (1969) The Fine structure and Chemistry of haemopoietic tissue. In, the Biological Basis of Medicine (edited by E. E. Bittar and A. A. Bittar) Academic Press, pp. 3-40.
- (4) R. Carriere (1969) The growth of liver parenchymal nuclei and its endocrine regulation. Intern. Review of Cytology. Edited by G. H. Bourne and J. F. Danielli, Vol. 25, Academic Press, N. Y. and London.
- (5) A. K. Sinha, S. Kakati and S. Pathak (1973) Extent of Ploidy in mammalian marrow and peripheral blood cells, Caryologia 26, 333-346.
- (6) H. Froberg and M. Schulze Schencking (1975) In vivo cytogetic investigations in bone marrow cells of rats, chinese hamsters and mice treated with 6-mercaptopurine, Arch.

The paper by Frohberg and Schencking (see No.6 in the above list) is recent, and the frequency of polyploid cells which they have reported in control mice and rats are 0.16% and 0.19% respectively.

With this background of well established scientific observations, the data of NIN and BARC as summarised in page 53 of the above said minutes (provided by the Minister is briefly re-examined before taking up certain other points of relevance.

The frequency of polyploid cells in the control rats used in the BARC experiments ranged from 0.00 to 0.362 (mean 0.2133 ± 0.0513) and 0.077 to 0.431% (mean 0.2488 ± 0.0408) in the treated series.

The frequency of polyploid cells in the control rats used in the NIN experiments ranged from 0.00 to 0.10 per cent. The NIN have presented that the frequency of polyploid cells in control rats in these series of experiments as 0.00% (see Table 8) page 14, Annual Report, NIN, 1974, and Table 5 of the paper by Vijayalaxmi and G.Sadasivan, Int. J. Radiat. Biol. 27 (1975), 135-142.

The frequency of polyploid cells observed in the control rats by BARC (i.e. 0.23 per cent) closely corresponds to 0.19% observed for control rats by Frohberg and Schencking (1975) and the geometric mean is ⁱⁿ the frequency range of 0.03 to 3.0 per cent normally reported in literature.

In an attempt to identify the cause of discrepancy

between the control values of the NIN and BARC, the Swaminathan Committee has observed (see page 53 section (b)) that in their (BARC) experiments a satisfactory number of cells have been scored whereas the NIN data have come from a relatively smaller number of cells (3000 cells per group as against over 20-30 thousand cells per group of BARC experiments and moreover, the standard error (SE) has not been provided.

The other finding of the NIN which has equally puzzled the Swaminathan Committee is regarding the transmission of the polyploid cells. The NIN's contention (see section 5, page 18 of the Annual Report, NIN 1974) is supported by their data (see Table 9, page 15, Annual Report, NIN 1974) which are as follows:

- (i) In the weaned group, rat progeny whose parents were fed irradiated wheat, the frequency of polyploid cells was 0.33% as against 0.03% in the control;
- (ii) Among the progeny of rats fed with irradiated wheat for 6 weeks, the frequency was 0.47% as against the control value of 0.03%;
- (iii) Among the progeny of rats fed irradiated wheat for 12 weeks the frequency of polyploid cells was 0.63% as against 0.03%.

It is difficult to explain the NIN's observation that the young ones born to parents fed irradiated wheat had significantly higher number of polyploid cells in the bone marrow at the time of weaning as compared to young ones born to parents fed unirradiated wheat. During the discussions on 24.6.1976, the Director, NIN, made it

clear that they did not mean genetic transmission but transmission through mother's milk. This would mean that the toxic substance supposedly present in the irradiated wheat enters the pathway of milk production in the mothers and then get transmitted to progeny which take mother's milk. What is even more interesting is that these factors transmitted from irradiated wheat to mother's milk induce polyploidy in the bone marrow of the pups.

Thirdly, the observation of the NIN (Table 9, page 15, Annual Report 1974) that the number of polyploid cells in progeny fed irradiated wheat increased from 0.33% at weaning to 0.47% at six weeks of feeding and to 0.63% at 12 weeks of feeding but the frequency of polyploid cells remained remarkably constant (at 0.03%) over a period of 12 weeks shows that aging of control animals upto 12 weeks does not cause any increase in the frequency of polyploid cells in the control animals. In subsequent publications (see Table 14, page 25 of Annual Report of NIN 1975), the NIN has observed 0.08 to 0.25 per cent polyploid cells in the control rats fed for 60 weeks on unirradiated wheat. The frequency of polyploid cells in the control was 0.12% whereas it was 0.89% in the treated rats. Weight control animals of the stock colony had an incidence of 0.08% polyploid cells in their bone marrow, while age control animals of the stock colony had 0.25% polyploids. The NIN has concluded that both age and body weight are among factor high influence the incidence of polyploid cells in normal animals.

The design of the experiment as well as the conclusions drawn are prone to several questions.

The so-called weight control animals, we understand, represent those maintained on 9% protein for 60 weeks and those maintained on 20% protein for 60 weeks. The frequency of polyploid cells in these two categories are 0.12% and 0.08% respectively. If their body weights are considered (Table 14, page 25, Annual Report, NIN 1975), they are 203.5 and 198.1 g respectively. Obviously, feeding on 9% protein has led to as much as, if not more, growth than the feeding on 20% protein. More important than this is the question whether 0.08% and 0.12% polyploid cells are really statistically different from each other. The S.E. values are given in the table of Enclosure III (page 23 of the material supplied by the Ministry) and it is seen that they are not significant.

Attention is drawn to the comments of the Director, Bio-medical Group (Enclosure II pages 16-21 of the material provided by the Ministry) that "the recent data from NIN on 60 weeks of feeding demonstrates that in the control animals according to their (NIN) techniques, the value can be anywhere between 0.12 and 0.25 per cent." The Director, NIN, has, in his clarification (see Enclosure II, pages 22-26 of the material provided by the Ministry), pointed out that 0.12% (obtained in long-term feeding studies extending to 60 weeks) is a considerably higher frequency than what is observed in short term (16 weeks) studies.

The NIN data presented in Table 9, page 19, Annual Report, 1974 and the data presented in Table 14, page 25, Annual Report 1975, suggest that up to 12 weeks of feeding unirradiated wheat the frequency of polyploid cells is 0.03% and then there is an increase from the 12th week (or unknown week) up to 60 weeks when the frequency becomes 0.12%, i.e. a four-fold increase. The increase owing to irradiated wheat over the same length of period is from 0.63% to 0.89%.

Attention is also drawn to Appendix II (Indian Council of Agricultural Research) page 39, last para "Rats fed confirms our earlier observation." Here the figures on the frequency of polyploid cells are again different from the figures presented elsewhere. For example, compare these figures with those on page 23 (Enclosure III of the material provided by the Ministry) or Table 14 page 25 of the Annual Report 1975; the control value in the Appendix II (page 39) is 0.13% (and not 0.12%) and the frequency of polyploid cells in the treated animals is 0.72% (and not 0.89%). Such mistakes suggest that not due care has been taken in collecting, handling and presenting the data.

Dr. Vijayalaxmi of NIN has published (Int. J. Radiat. Biol. 27, 283-285, 1975) the results of a study in which irradiated wheat was stored for 12 weeks before being fed to rats and the time-course of appearance of polyploid cells with increasing duration of feeding freshly irradiated

Int. J. Radiat. Biol 27, 1975) in animals fed unirradiated wheat, freshly irradiated and stored irradiated wheat was 0.04%, 0.58% and 0.10% respectively. The finding is that when irradiated wheat is stored for a period of 12 weeks before being fed, no increase in the incidence of polyploid cells is seen. The interpretation thereof was that during irradiation of wheat some 'toxic substance(s)' is/are formed, the concentration of which falls during storage.

The same paper contains data (table 2) which show that only after six weeks of feeding freshly irradiated wheat, a significant increase in the incidence of the polyploid cells is seen. From the sixth week to the tenth, this frequency approximately doubles.

THE MOST STRIKING ASPECT OF THE VARIOUS NIN REPORTS ON THIS SUBJECT IS THAT THEIR STUDIES HAVE SHOWN A FREQUENCY OF POLYPOID CELLS IN THE CONTROL RATS WHICH IS SURPRISINGLY BELOW THE ESTABLISHED LEVEL OF ABOUT 0.20 per cent.

Realizing this point, the Swaminathan Committee had suggested NIN and BARC should jointly conduct studies in order to settle the frequency of polyploid cells in the control animals of BARC and NIN. The studies were also so designed as to unravel any influence of the differences in the techniques employed by NIN and BARC.

NIN-BARC joint studies were also expected to throw some light on the possible influence of the two locations viz. the animal house of the NIN and the animal house of BARC on the incidence of polyploid cells in the bone marrow of wistar rats.

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The experimental design for the NIN-BARC joint studies was as follows: Eight rats each from the BARC and NIN colonies were taken for these experiments. Of these 8 rats of BARC 4 were processed by the BARC technique at BARC (animal nos. 11, 12, 15 and 16). The remaining 4 were processed by the NIN technique (animal nos. 3, 4, 7 and 8). Of the 8 NIN rats 4 were processed by the NIN technique (animal nos. 1, 2, 5 and 6). The remaining 4 NIN rats were processed by the BARC technique (animal nos. 9, 10, 13 and 14). Every slide was planned to be scored by both Dr. Vijayalaxmi of NIN and independently by Shri George of BARC.

The high lights of the joint studies conducted by the BARC and NIN as summarised by NIN are presented in enclosure II pages 63-64 of the material provided to us by the Ministry. However, these comments must be read together with the comments of the Director, Bio-medical Group, BARC (enclosure II, pages 16-21 of the material supplied by the Ministry). We find these comments together with the original material most instructive and present below our analysis of the same.

Although it was intended that all the four slides from each of the 16 animals selected for the joint study would be scored by both Dr. Vijayalaxmi and Shri George, in practice the slides of 4 animals were not scored by Shri George, further 2 slides were broken. Of necessity therefore the analysis had to be limited to the remaining 46 pairs of observations, 4 from each of the 10 rats

(nos. 6, 9, 10, 13 and 14 from NIN colony and 7, 8, 11, 12, 16 from BARC and 3 from rat nos. 3 and 15 each). Since the 4 slides for each animal constitute the whole aliquot extracted from the animal's bone-marrow and are not independent replicate we have analysed the data on a rat per slide basis and find (i) None of the sources of variation gives a mean square for the frequency of polyploids that is significantly larger than the error mean square in the case of NIN animals; by contrast the mean square due to animals comes out to be significantly larger than the means square for error in the case of BARC animals. Further a sub-division of the mean square due to rats shows that by far the largest contribution to this mean square is made by differences in the two techniques. This is true both of NIN as well as of BARC rats though the mean square due to techniques is not significantly larger than the error mean-square in the case of NIN rats; (ii) the mean square for error variance based on the interaction of observers with animals is 10 times as large in the case of NIN as in the case of BARC rats; (iii) Expressed in terms of the different components which go to make up the model implicit in the analysis of variance viz. that an observation y_{ijk} can be represented by

$$y_{ijk} = \mu + x_i + \lambda_j + \psi_{ij}$$

where x represents the deviation of the true value from the general mean μ ,

λ represents the contribution of the observer's bias and ψ the interaction, $E(x_i) = 0$, $E(\lambda_j) = 0$ and

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$E(\epsilon_{ij}) = 0$ and

$$E(x_i^2) = \sigma_x^2, E(L_j^2) = \sigma_L^2 \text{ and } E(\epsilon_{ij}^2) = \sigma_\epsilon^2$$

with the variance of an observation given by

$$V(L_{ij}) = \sigma_x^2 + \sigma_L^2 + \sigma_\epsilon^2 = \sigma_y^2 \text{ say}$$

we find that by far the largest component of the variance of an observation is the error variance in the case of NIN animals contributing some 70% of the total variation. As against this it is σ_L^2 for animals which is found to make the largest contribution to the variance of an observation in the case of BARC animals representing over 90% of the total variation. The implication of this finding is that the co-relation between the observations recorded by the two observers on the same slide in the case of BARC rats is very high but is poor in the case of NIN animals suggesting that subjective elements arising from variable bias affects the scoring made by an observer on the NIN material. Some evidence of this can be seen in the variable count of the number of polyploids made on NIN material. It would appear that an observer's ability to identify and count the polyploids was affected by his or her intrinsic tendencies whenever he or she scored the slides from the NIN rats. This in its turn has increased the magnitude of the error variance.

Finally it needs to be repeated that of all the factors influencing observation, the technique influences the most.

In terms of the model these findings imply that if we have observers of the type represented by

Dr. Vijayalaxmi and Shri George from the two laboratories and techniques typical of those developed at NIN and BARC we can expect the frequency of polyploidy in the bone marrow of control animals to have an error variance of the order of 0.015 giving for the standard error per rat a value of 0.12%. For a mean value based on six rats, which is the number used in the experiments to test the differences between frequencies of polyploids in irradiated and unirradiated wheat fed rats, this means that we can expect a standard error of the order of 0.05%. This is a little smaller than the value one would expect based on BARC data for their control rats and can therefore be safely used for judging the limits of the range of the frequency of polyploidy in the control animals. NIN also has pointed out that the incidence of polyploidy can be 0.12 per cent (vide Ministry's report, p.23). Considering that we have if anything underestimated the standard error and the colony of rats selected for the joint study was homogenous, it would appear that the NIN value of 0.12% is not inconsistent with the value recorded by BARC on the control of animals or that of 0.19% reported in the literature.

As against this picture we find that the incidence of polyploidy in the case of treated series reported by NIN is very high, being 0.6% significantly larger than the corresponding value of 0.0% reported in their experiments on the control rats. One of us (Dr.P.C.Kesavan) has recounted the slides previously enumerated by Dr.Vijayalaxmi

in 1973 using the method of complete enumeration for the purpose. The results are set out in Table 1. It will be seen that the incidence of polyploidy among the treated series based on the complete count comes out to only 0.14%, nowhere near 0.6 reported by NIN. The same table also shows the results of the corresponding counts previously made by Dr. Vijayalaxmi in 1973. It will be seen that the mean value for the incidence of polyploids based on her previous counts works out to 1.0%, considerably in excess of their earlier figure of 0.6% and 7 fold as high as the value based on the complete count of the same slide carried out by us. The comparison demonstrates that the NIN observations on the frequency of polyploids in rats are not only subject to a larger error variance but also suffer from bias.

We have gone into some detail to examine the cause of the high upward bias in the treated series. We find that the so called random method used by Dr. Vijayalaxmi is not random in the known sense of the word. She chose for a starting point a value on the Y-axis of the slide at her own sweet will without using any chance mechanism. A random method as the word connotes must be based on the rules of chance using either a table of random number or some other suitable mechanism to ensure that every unit area on the slide gets an equal chance of being included in the sample for enumerating all the scorable cells and polyploids. This can only be achieved by choosing for the starting point a XY co-ordinates at random in the sense explained above. Since Dr. Vijayalaxmi had not

marked the location of the starting point using XY co-ordinates for the purpose we gave her a test to see how she went about selecting the starting point of the 100 consecutive scorable cells which she enumerated for determining the frequency of polyploids. This test clearly showed that she tended to select for the Y co-ordinate a figure in the middle range of the Y-axis of the slide. We have tabulated the frequency distribution of the random starts in table 2. It will be seen that she has over-sampled the central left portions of a slide with a probability 2 to 3 times as large as the surrounding areas. We have compared the count made by her in these tests with the counts previously recorded by her in 1973 using the same method of scoring 100 consecutive cells and find that the two differ enormously from each other (vide Table 3). The test serves to give an idea of the large differences that can be expected to occur in repeated samples using her technique of enumerating 100 consecutive cells.

Normally if the method used by Dr. Vijayalaxmi had been objective we would not have obtained any such large difference. Even in the biased selection the differences would not have been so large as observed if the polyploids had been distributed evenly all over the slide. We find however that not only are the polyploids not distributed evenly but also they cluster together as can be seen from Table 4 showing the serial number of the scorable cell at which a polyploid was observed in the complete count made by us. It would appear that Dr. Vijayalaxmi tended

to select a line on the Y-axis which showed a large incidence of polyploids overestimating the incidence of treated series in the process. Table 5 gives the results of the counts made by her on slides from B/KC which have been previously completely enumerated. Once again we find that there are large difference between the two.

We believe that we have produced enough evidence in the analysis reported above to show that NIN results suffer from considerable bias, when they are corrected for bias they cannot be considered as inconsistent with those reported by BARC. The differences between NIN and BARC do not therefore lie so much in the adoption of this design or that or this method ^{of} analysing variation or that or whether differences are significant at 5% or 6% level of significance but in our view they lie mainly in the faulty and biased selection of the sample. As an example the adoption of a random sample design by NIN using body weight to allot males to the different blocks is criticised by BARC on a priori grounds as totally unjustifiable. The results show that BARC's objection is valid in as much as the analysis shows that the variation actually removed by blocks is of an order of smaller than that of the residual variance. The implication of BARC's remark that the device of randomised blocks is used to artificially inflate the level of significance by NIN is thus not true; if anything the NIN has lost on precision of the mean by using the randomised block design. Other points of differences between BARC and NIN made in the

comments by the Directors of the respective laboratories equally make little difference to the major conclusions reported by the 2 laboratories. As we have said above the main discrepancy arises from the biased and faulty selection of the sample.

One way of getting over the bias in selection is to enumerate the slides completely as BARC has done. This is of course an expensive and time consuming method. But it is far better to err on the safe side than go about the way the NIN did. On the other hand it should not be difficult to devise a sampling technique that gives an estimate of the number of polyploids and of the scorable cells on each slide within a given margin of error. The recent decision by NIN to increase the size of the sample from 100 to 200 consecutive scorable cells per slide is a step in the right direction but even this will not compensate for the faulty and biased method of selecting the starting point to locate a grid around it. Again it is important that the sub-sampling of a slide must be done in 2 or more sub-samples and not by adopting one single line of 100 or 200 consecutive cells as NIN has done. There must be provision for independent area samples with independent random starts for each in the manner explained above to ensure that the method is free from bias and the same can provide an estimate of the mean count of polyploids and scorable cells with given precision.

Workers from BARC (George et al, Fd. Cosmet. Toxicol. 14, 1976) have conclusively shown that cytological analysis

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of bone marrow showed no significant difference in the frequency of polyploid cells in the rats fed non-irradiated or irradiated wheat diets, even when treated wheat was fed to the rats within 24 hours of irradiation.

Lastly we should like to point out that the NIN adopts a very unconventional practice of leaving the slides without cover glasses on and therefore these slides could not be easily studied under the oil immersion. The difficulty is that the cells may begin to move under the impact of oil and they are bound to be lost when the slides are cleaned afterwards with xylene and tissue paper. Unaware of this fact that there were no cover glasses, we had most of the cells of 4 slides belonging to 2 animals erased out. What we cannot imagine is how the NIN could have scored these slides.

Polyploid cells in the bone marrow of mice fed irradiated wheat

Studies similar to those carried out in rats were undertaken in Swiss albino mice. Thirty weaning albino Swiss mice were divided into three groups of five males and five females each and given one of the following three diets: (1) 70% unirradiated wheat; (2) 70% freshly irradiated wheat (wheat was used within 20 days of irradiation) and (3) 70% stored irradiated wheat (wheat was stored at 4°C for three months after irradiation). All diets provided 9% protein. At the end of 12 weeks on these diets, all animals were sacrificed and chromosomal preparation from bone marrow cells were made. One

thousand cells were examined for each animal (i.e. 200 consecutive cells per slide and five slides per animal) to determine whether numerical aberrations were induced by irradiated wheat. The relevant data are presented in Fig. I page 24 of the Annual Report on NIN 1975 and also Table 4 page 70 of the material supplied by the Ministry. The incidence of polyploid cells in mice fed freshly irradiated wheat, unirradiated and stored irradiated wheat were 0.40%, 0.09% and 0.07% respectively. It has already been pointed out that the normal frequency of occurrence of polyploid cells in the bone marrow of mice is 0.17 per cent. Furthermore, our comments regarding the NIN method of scoring and analyses of the data on the incidence of polyploid cells in the bone marrow of rats also apply to their studies on mice. There is little need to repeat all these arguments, and this committee concludes that the freshly irradiated wheat does not induce polyploid cells in the bone marrow of mice either. NIN studies on the mice and rats taken together fail to demonstrate that the irradiated wheat produces numerical variations in the chromosomes of the bone marrow cells.

3. Effects of freshly irradiated wheat on meiotic chromosomes in mice

The data under discussion are contained in Table II, page 68 of the Annexe supplied by the Ministry of Health and Family Planning as well as in Table 15, page 29 and Figure 2, page 27 of the NIN Annual Report, 1975. The conclusions of the NIN (page 26, Annual Report, 1975)

are as follows:

(i) There were no differences in the incidence of either polyploid cells or cells having X and Y univalents between the groups of mice fed unirradiated wheat or freshly irradiated wheat or stored irradiated wheat,

(ii) The incidence of aneuploid cells (cells with less than the normal number of 20 bivalents) are twice as high in mice fed freshly irradiated wheat as compared to those fed unirradiated wheat or stored irradiated wheat. This difference was statistically significant. There was, however, no difference between mice fed the unirradiated wheat and those fed stored irradiated wheat. NIN data showed that the numerical variations of aneuploidal and euploidal types constitute 11.0 ± 0.77 plus $4.3 \pm 0.56\%$ respectively in the spermatocytes of the control mice in the NIN studies. In their studies the frequencies of these two types for mice fed freshly irradiated wheat were 22.2 ± 0.98 plus $5.1 \pm 0.76\%$ respectively. It occurred to this Committee that these male mice ought to have been highly sterile but this was not the case. In fact these males exhibit capacity for very successful mating, almost similar to those of BARC. The Director, NIN, has pointed out (page 24, Item III, Annexure III of the Appendix supplied by the Ministry of Health and Family Planning) that these mice had been kept only on 9% protein level - i.e. clearly below the requirement level and it is not known whether this had contributed to 11.4% aneuploidy. He has added that the pregnancy rate in the females mated

with these males was between 60 and 70% - a value much lower than the figure of more than 90% reported by BARC in their reproductive studies in normal mice. We have checked up the BARC data on the pregnancy rates in mice fed stock diets. The pregnancy rates are in the range of 60 to 70% only. In this context it also occurred to us to verify as to whether such a major change in the chromosome complement could lead to an increase in intra-uterine deaths. This Committee therefore examined the NIN control data on the percentage of intrauterine deaths in mice (Table 5, page 31 and table V of page 7 of the Annexes provided by the Ministry of Health and Family Planning). These data do not show that these males when mated to females caused an increase in the number of dead implants. In fact their frequency of occurrence is very low as compared to other stock animals reported in the literature.

Our survey of literature does not suggest that variations in the chromosome numbers in the meiotic cells occur in the frequency range reported by the NIN. The type of aneuploidy (cells with 19, 18, 17, 16, 15 and less than 15 bivalents) reported by the NIN is clearly nullisomic. This type of nullisomic, involving the loss of pair or pairs of homologous chromosomes, could be expected to arise, from non-disjunction in the anaphase I; but if this is the mode of origin of nullisomics at one of the poles, there should be corresponding additions of pair or pairs of bivalents in the opposite pole. However, on enquiry, the NIN workers stated that such cells with 21, 22, 23,

24, 25 and greater than 25 bivalents in about 11% of the spermatocytes of the control mice and 22.2% of the spermatocytes of the mice fed freshly irradiated wheat have not been observed by them.

Keeping these points in view this Committee examined the slides of meiotic preparations in male mice when this Committee visited NIN, Hyderabad in November 1975.

Dr. Vijayalaxmi of NIN mounted one of these slides on a microscope and focussed a cell which she believed to contain 19 bivalents, with the deletion of 1 bivalent. One of us (Dr.P.C.Kesavan) identified that this cell had 18 bivalents and 1 quadrivalent. This was pointed out by us to Dr.Vijayalaxmi and the Director, NIN.

A few other cells which had only 18, 17, 16, 15 and less than 15 bivalents were carefully examined by us in and around these cells. It became quite obvious that 2, 3, 4, 5 and more than 5 bivalents had been extruded from these cells. Secondly, it appeared that these cells had fewer bivalents, whereas the extruded bivalents could be traced at different distances around these cells. It is very likely that improper air-drying could have caused these artifacts. Furthermore, that these cells with a complement of less than 20 bivalents were only artifacts is supported by the absence of the cells with correspondingly more than 20 bivalents. The question of non-disjunction of bivalents, therefore, does not arise.

This Committee, having found out what caused the artifacts in the NIN studies, advised the NIN to withdraw

these data in November, 1975. During our second and final visit to NIN, Hyderabad, on 24.6.1976 this Committee was distressed to learn that Dr. Vijayalaxmi, instead of withdrawing the paper as per the advice of this Committee, had got the paper accepted for publication in an international journal. When questioned again, she told that all the cells with less than 20 bivalents could be due to translocations. This Committee pointed out that such high frequency of translocations have never been observed by any workers in the field and also it referred to the work of Leonard and Deknadt, (Mutation Research, 9, 127-133, 1970), who had observed one quadrivalent (chain type) out of 20,700 spermatocytes in the control mice. It must also be pointed out here that Prof. G. Sadasivan who had been earlier associated with the work of NIN was also present during our discussion on 24.6.76 and he volunteered an information that he too had warned Dr. Vijayalaxmi that whatever she observed were cases of extrusion of bivalents from broken cells and not true aneuploids. With respect to the NIN observations that the frequency of such aneuploid cells was double in male mice fed freshly irradiated wheat as compared to those fed unirradiated wheat, this Committee believes that the NIN had been a victim of preconceived notions.

This Committee, in order to impress upon the artifactual nature of their findings, pointed out that occurrence of 11% aneuploids in spermatocytes in the control and 22.2% aneuploid cells in the treated mice would lead to degeneration of approximately 44% of spermatozoa in the control and approximately 88% of the spermatozoa in the

treated mice. But, very surprisingly, the reproductive performance of these male mice is as high as those of the male mice which do not have such meiotic abnormalities. If the NIN findings of aneuploids of the magnitudes reported by them are true, their male mice would have been totally sterile. Our arguments are also based on stochastic considerations.

Under these circumstances, this Committee once again appeared to NIN on 24.6.1976 to withdraw these data as it would otherwise be a discredit to Indian Science. However, NIN kept silent about it. This Committee strongly recommends the rejection of these data as they are nothing but artifacts

4. Effects of irradiated wheat on germ cells survival in male mice and rats

There are no data from BARC. Considering the inherent intricacies involved in the assessment of the effects of toxic substances on germ cell survival, it would be prudent to avoid these studies and rely to a greater extent on the data on fertility index alone. It is established beyond doubt that a reduction in the germ cell counts results in marked sterility in the males. The essence of the argument is that the study of germ cell survival involves huge amount of labour, time and resources but the value of the information gained is very marginal as compared to the studies of the assessment of the fertility index of the animals.

However, the NIN has carried out these studies and presented the data in Table 11 (Germ cell survival in

rat testis) on page 17 of their Annual report 1974 and in table 16 (Germ cell number in male mice fed irradiated wheat) on page 30 of their Annual Report 1975. The conclusions of the NIN are as follows:

(i) There was a significant reduction in the number of germ cells in the malnourished rats (Annual Report of NIN 1974). This implied that malnutrition per se incapacitates the males to sire offspring.

(ii) The number of type-A and type-B spermatogonia were significantly lower in mice fed freshly irradiated wheat as compared to those fed either unirradiated or stored irradiated wheat.

(iii) There were no significant differences between mice fed unirradiated wheat and stored irradiated wheat. These data again are similar to those observed in rats (page 26, Annual Report, NIN 1975).

An unfortunate omission in the Annual Report 1974 of NIN is the technique used to obtain the germ cell counts in rats. Furthermore, this omission becomes more prominent when one reads the Annual Report 1975 of NIN, wherein it is stated (page 26) "the technique followed was similar to that followed earlier for rats (Annual Report 1974)". Hence, this Committee had to resort to correspondence and oral discussions with the Director and staff of the NIN to get to know the technique employed in these studies. This clarification received from the Director, NIN was very useful in drawing certain conclusions based on the information available.

in literature.

We find from the Section IV of the enclosures II (pages 16 to 21) and III (pages 22 to 26) of the material provided by the Ministry of Health and Family Planning the Director, Bio-medical Group, BARC and the Director, NIN had differences of views regarding the observational basis of 1:1:1 ratio for type-A spermatogonia: Type-B spermatogonia: Resting Spermatocytes which are now known as preleptotene cells.

In view of the fact that the explanation offered by the Director, NIN, on the question of ratios of type-A to type-B to Resting Primary Spermatocytes (RPS) was not reconcilable with known facts in literature, the Director, Bio-medical Group, BARC wrote to 2 outstanding authorities Prof. Oakberg of Oakridge National Laboratory, USA and Prof. Clermont of McGill University, Canada. The Committee had an opportunity of examining their replies. Though there could be some disagreement with regard to actual ratios of type-A to type-B, both the experts in the field have advised that the ratios of type-A to RPS will normally be 1:4-6. In order to achieve the correct relationship, the normal technique followed is to enumerate the peak values of type-A, type-B and RPS in stages II, V and VII respectively in proportions of tubules representative of testis.

It was evident during discussion and earlier correspondence that in order to justify the ratio of 1:1:1, NIN had adopted a very simplistic model which is far

removed from the models developed by experts in the field like Cakberg, Clermont, Huckins and Monnessi. The Committee had to examine this aspect in depth in order to satisfy itself that the quantitative estimate obtained by the investigators are acceptable before proceeding to make any comparisons. The Committee also observed that in their publications (International Journal of Radiation Biology, Vol.29 table 2, page 96, 1976 and table 11, page 17, Annual Report NIN 1974) in the depleted animals (malnourished) the type-B cells were significantly lower in numbers than type-A cells. This is very surprising in view of the fact that type-A cells could give rise to type-B cells through a series of divisions and therefore the number of type-B cells should at least be equal to type-A even if one has to account for degeneration. The explanation for this observation was sought on 24.6.1976 when the Committee visited NIN, Hyderabad, but it was not answered.

In addition the same publication showed that while statistical differences existed for type-A cells and RPS between the control and experimental groups, no differences were observed for type-B cells. With respect to the short term study-2 on rats the NIN has concluded (see item 3 of, 11.5, 11.2 of p.13 of Annual Report NIN 1974) as follows: "There were no significant differences in the mean litter size, birth weights or weaning weights between the groups of animals fed irradiated and unirradiated wheat." In view of the concepts given above, such a phenomenon cannot occur. This only reinforced the belief of this Committee

The Committee concludes that no significance can be attached to these data.

5. Dominant lethal studies in rats and mice

Dominant lethal tests (DLT) in mice and rats have been carried out both by NIN and BARC. The NIN data show that the freshly irradiated wheat significantly enhances the frequency of intrauterine deaths (variously referred to as dead embryos (DE), dead implantations (DI) etc.) in rats and mice.

The BARC data, however, did not show such effects. For detailed analyses it is necessary to consider the studies of NIN and BARC separately.

(a) NIN studies on rats: Males reared either on a diet of 18% pr-otein (to form the well-fed group) or a diet of 5% protein (to form the depleted group) for 8 weeks are divided into 3 groups of 6 males each and fed one of the following diets containing (i) 70% unirradiated wheat (ii) 70% freshly irradiated wheat and (iii) 70% freshly irradiated wheat plus casein. The first two of these diets provide 9% protein while the third provided 18% protein of which 9% came from casein. The animals of these 3 sub-groups were fed for 12 weeks on the respective diets.

The mating scheme consisted of mating each of the six males from each of these diet groups to 3 virgin females per week for 4 weeks. This means that 72 females per dit group for 4 weeks comprised the test population.

switched over to unirradiated and irradiated wheat respectively. In both the cases, the statistical significance ($P < 0.01$) is evident only when the data of mating weeks III and IV are pooled. If data of each of the four weeks are pooled together, the increase in the mutagenic index induced by irradiated wheat is significant at $p < 0.05$ for the well-fed group of rats and at $p < 0.01$ for the depleted group of rats. In order to explain the significant increase in the mutagenic index associated with males mated to virgin rats in third and fourth weeks but not in the first and second weeks (after withdrawing these males from irradiated wheat and putting them on stock diet), the NIN made the point that "spermatids were more sensitive to the test substance than the spermatogonia themselves", (see page 15, para 1 of the letter of Dr. S.G. Srikantia, Director, NIN, to the Chairman, Atomic Energy Commission vide letter No. D.11/1/2887 dated May 24, 1974.

This contention of the NIN makes it sound as though their experimental design of feeding the male rats with irradiated wheat is either acute or sub-acute. In fact, this is not so. It is pointed out that the male rats were fed irradiated wheat for 12 weeks; this is unmistakably a 'chronic' treatment with whatever toxic substances, if any, are present in the irradiated wheat. Since the spermatogenic duration is of 60-70 days in rats (see page 90, Table 1 of the book entitled, "The testing of chemicals for carcinogenicity, mutagenicity and teratogenicity", 1973 published for the Ministry of Health and Welfare, Canada),

all the stages starting from the stem cells, spermatogonia A to spermatozoa in epididymis would have come into contact with the toxic principle, if any, in the irradiated wheat and then by the end of 12 weeks, all the affected cells (no matter at what stage they were sensitive and were affected) would have reached the epididymis ready for ejaculation.

So, considering the design of these studies, one should observe an increased mutagenic index (if it occurs) in the very first (i.e., the mating week I after withdrawal of the male rats from irradiated wheat on which they were fed for 12 weeks) week of mating even. It is difficult to assume that the spermatids which alone were more sensitive to the mutagenic effects of irradiated wheat remained as spermatids and did not begin to mature and enter the epididymis until the males were withdrawn from the irradiated wheat. It is well established that "sensitivity spectrum analysis" cannot be carried out using chronic treatment with mutagens and suspected mutagens. Hence, the view already expressed by Dr.K.Sundaram, Director, Bio-medical Group, BARC, Bombay (see page 21, annexe to the Report of Swaminathan Committee) that, "from the mutagenicity effect point of view the sperms released on the 13th week (i.e. mating week I) would carry the sum of all the effects on the preceding stages from which these sperms are derived" is indeed valid.

If the experimental design involves acute administration or sub-acute administration of a mutagenic substance, it may be possible to obtain a peak in the mutagenic

index in a certain later mating (not the first mating) corresponding to the time taken for the affected cells of earlier stage to mature and reach the epididymis for ejaculation and fertilization. In other words, if cells of an earlier stage are affected by an 'acute' treatment with a mutagen, then the induced mutations would become observable only when they mature and reach the epididymis. The problem with chronic feeding is that the duration of treatment exceeds the duration of spermatogenesis and therefore, epididymis will contain all the 'affected' as well as unaffected spermatozoa. Since the 'affected' and the unaffected sperms have an equal probability of fertilizing an egg, the increased mutagenic index associated with the 'affected' cells, if any, should be observed in the first mating itself.

That this was not, however, the case with the observations of the NIN indeed puzzled us; in fact, these observations, in a way, weaken their very view point of mutagenic effects of irradiated wheat on well-fed and depleted rats. What is even more interesting is that the NIN, having become aware of the weakness of their argument had withdrawn this point in their recent publication in Int.J. Radiat. Biol. 29, 93-98, (1976). This Committee pointed out on 24.6.75 that their earlier reference to sensitivity spectrum following chronic feeding experiments had not found its place in their above-said paper in order to avoid confrontation with the referees and if this was so, it was quite unethical on their (NIN) part.

It should be pointed out although the NIN had claimed significance of their data at $p < 0.05$ to $p < 0.01$, this was not sustained when one of us (PVS) reanalysed their data. In fact, one of us (PVS) had written to Dr. Srikantia, the Director, NIN, on August 20, 1974 that the observed 'F' values are not significant at the conventional level of 5 per cent, but this probability is of the order of 6 per cent for differences between diets both in the case of well-fed and depleted groups. In the same letter, it was also pointed out that the weakness of Dr. Sundaram's analysis was that he combined the main effects due to weeks (3 d.f) and the interactions between weeks and diets (3 d.f) under one item with 6 d.f. against weeks and hence he did not find that the main effects were significant.

Asked to comment upon it, Dr. Sundaram stated that the experiment does not conform to randomized block design since weight of animals cannot be taken as a criterion and also no significance can be attached to the observed 6 per cent level of significance in view of tremendous biological variability in the female animals over which the experimenter has no control.

We also believe that body weight cannot be included as a criterion for the randomized block design, since it would imply that dominant lethal mutations vary with body weight, which is not true.

Furthermore, in all the mutagenicity testing, using the Dominant lethal test there is an agreed protocol that the effects induced by an agent should at least be 5-fold

to deserve any serious consideration. This is in view of the fact that the ratio of minimum value to the maximum value even in the control or stock can be as high as 5 times - in the published work in this field. This is seen even in the data on the control animals of the NIN experiments.

Conclusions: Besides the points mentioned above, it is necessary to point out that in these experiments, the NIN investigators did not find any differences among the total implantations or the litter size between the control and experimental diet fed groups (vide Annual Report NIN 1974). Since the total implantations is the sum of live births and dead implantations it is logical to expect that if one parameter is affected, it should be reflected in other related parameters as well. As this has not been demonstrated, in their (NIN) experiments, the Committee concludes that the statistical significance of even 6 per cent carries no weight. The Committee would also like to record that withholding of part of their observations which would go against their acceptance and therefore its publications, is to say the least "not good scientific tradition."

(b) NIN studies on DLM in mice:

Twenty seven weanling male albino Swiss mice were divided into three groups of nine each and were fed (i) unirradiated wheat, (ii) freshly irradiated wheat, and (iii) stored irradiated wheat diets respectively

was no depleted group of animals. At the end of twelve weeks, each male was mated with three virgin females per week from the stock colony for four consecutive weeks.

The wheat diet was replaced with stock diet during the 4 weeks of mating. All females were sacrificed 13 days following the mid week of their caging and presumptive mating. Their uteri were screened for live and dead embryos. These data are found in figure 3, page 28, Annual Report, NIN, 1975 and Table 4, page 31 and Table V page 71 of the annexes to the Report of Swaminathan Committee.

It must be pointed out that the mean values given in figures 3, page 28, Annual Report NIN, 1975 and Table V, page 71 of the annexes to the Report of Swaminathan Committee differ with those of Table 5, page 31 of the annexe to the Report of Swaminathan Committee.

It was concluded that the mutagenic index was higher in freshly irradiated wheat group as compared to unirradiated ($P < 0.025$) and stored irradiated wheat groups ($P < 0.05$).

In passing, it is mentioned that in figure 3 (page 28, Annual Report NIN, 1975) the term, 'per cent mutagenic index' is used; this is incorrect in view of the fact that "mutagenic index" is calculated as:

$$\frac{\text{Number of dead embryos}}{\text{Total implants}} \times 100 \text{ for each female}$$

As is the case with rats, these experiments on mice also show that the mutagenic effects of irradiated wheat are shown to occur in mating week III. This observation is difficult to explain in view of the fact

that the irradiated wheat was fed for 12 weeks, whereas the duration of spermatogenesis is less than 9 weeks in mice, and hence the experimental design conforms to 'chronic treatment'. Under these circumstances, the mutagenic effect of the irradiated wheat, if any, should be observable in the mating week I itself.

The other statement of the NIN (page 31, para 1, Annual Report, NIN, 1975) that "the increased intrauterine deaths in this group may partly be related to the increased incidence of meiotic chromosomal abnormalities found in these animals" deserves scrutiny. With reference to the effect of irradiated wheat on rats, NIN had already reported (see item 3 of 1.5 1.2 short-term study-2 on page 13 of the material provided by the Ministry) that "there were no significant differences in the mean litter size, birth weights or weaning weights between the groups of animals fed irradiated and unirradiated wheat". Furthermore, our analysis of the meiotic data (see Appendix III of the NIN reveal that artifacts alone appear to be the cause of all the fuss and confusion.

When one looks at the meiotic data and the LMA data of the NIN, it may appear to follow a certain pattern, but, in fact, this is not so. The fertility index, meiotic data, germ cell survival data and the data on dominant lethal mutations have all their own weaknesses, in terms of experimental design, biological concepts as well as the statistical analysis. Furthermore, these are all inconsistent with each other. For example, the

data on the mean litter size and dominant lethals in rats and mice are evidently mutually contradictory; germ cell survival data and meiotic data are also contradictory with the data on the fertility index of the control and the experimental groups of animals.

(c) BARC data on rats:

Studies for testing dominant lethal mutations in rats fed irradiated wheat (75 kR) were done in three phases by BARC. In phase I, wheat was irradiated everyday with 75 k rad and included in the diet of animals for seven days. In phase II, animals were fed on wheat irradiated every week, the feeding being continued for six weeks. Phase III was of the same regime excepting that the feeding was continued for twelve weeks.

The detailed procedures as well as the data pertaining to these studies are presented in Enclosure IV (pages 72-81) of the Report of Swaminathan Committee.

It is noted that irradiated wheat or unirradiated wheat constituted about 70 per cent of the diet. After a specified duration of feeding, three virgin female rats were placed with each male and were removed after one week. A fresh batch of virgin females was used in every subsequent week and the sequential weekly matings were continued upto 5 weeks. Female rats were sacrificed on 18th day after their initial caging with the male and examined for dead and live implants in the uterus and corpora lutea in the ovaries.

Data on pregnancy (part A tables for each of the

three phases in the material provided by the Ministry) of control animals are in the range of 62.1 to 94.9 with a mean around 80 per cent and of experimental in the range of 61.5 to 94.9 per cent with a mean around 82.0 per cent. Thus, there is no evidence of any adverse effect of irradiated wheat on the fertility index. On the basis of the information provided by NIN (column 3 of 11.5.1.2, short-term study-2, page 13 of Annual Report, 197 we would accept their own conclusions that there were no significant differences in the mean litter size, birth weights or weaning weights between the groups of animals fed irradiated and unirradiated wheat.

With respect of corpora lutea, percentage dead implants etc. also, the control and the experimental groups exhibited almost the same range of variability and there was no question of any statistical significance of the differences at all. BARC has concluded that irradiated wheat does not appear to have any effects on the frequency of pregnancy and percentage of intrauterine deaths in rats.

Our attention was also drawn to a paper published by the BARC (Chauhan et al. Int. J. Radiat. Biol. 28, 1975, 215-223) on the dominant lethal mutations in third generation rats reared on an irradiated diet. The irradiated diet (0.2 Mrad and 2.0 Mrad) was given only after storage for 3-4 weeks at 4-6°C. In these experiments, the third generation male rats of the multi-generation studies on the effects of irradiated

food failed to exhibit any significant change in their fertilization capacity. The only difference observed was a reduced implantation rate ($P < 0.05$) observed in the second weekly mating of animals given the irradiated (0.2 Mrad) diet. No such effect was observed in any of the other weekly matings of animals given food irradiated at 2.5 Mrad. It is well established that a reduction in the number of total implants is indicative of the occurrence of pre-implantation losses (Epstein, S.S. and Röhrborn, G. 1971, Nature, Lond, 230, 459) and such pre-implantation embryonal deaths can be induced by numerous non-genetic factors (Bateman, A.J. and Epstein, S.S., 1971, Chemical Mutagens, Principles and Method for their detection, Vol.II, edited by A.Hollaender (New York: Plenum Press, p. 541) and can, therefore, at best be considered as a concomitant measure of sub-ordinate importance but not as an alternate parameter to post-implantation loss for the evaluation of mutagenicity in mammals. However, the post-implantation loss, which afford a direct measure of mutagenicity determined either as dead implants per pregnant female or as percentage of total implants, remained comparable among different groups. Furthermore, the cumulative percent frequency distribution of dead embryos did not, change as a consequence of the rats' eating irradiated foods.

Although not of direct relevance to irradiated wheat, it is to be pointed out that, Renner, Grunewald and Ehrenberg - Kiechebusch (Humangenetik, 18, 155, 1973)

failed to find evidence of any increase in dominant lethal mutations in five successive generations of rats given a diet containing 35 per cent milk powder irradiated at 4.5 Mrad and also in mice fed an irradiated (4.5 Mrad) whole diet for 8 weeks. Thus these studies provide almost conclusive evidence of the lack of induction of dominant lethal mutations in the third generation of male rats fed irradiated diets.

We understand that one more paper entitled "Evaluation of freshly irradiated wheat for dominant lethal mutations in wistar rats" is under publication.

(d) BARC studies on Mice:

Our analysis is based on the paper entitled "Dominant lethal mutation on male mice fed irradiated diet" (Fd. Cosmet, toxicol 13, 433-436, 1975) by Chauhan et al. From the age of 4-5 weeks, three groups of swiss male mice were fed on a stock ration, or an unirradiated or irradiated (2.5 Mrad) test diet for 8 weeks, after which they were all given the stock ration and kept for mating with virgin untreated females (9-11 week old) for 4 consecutive weeks. The females were autopsied 10-11 days (about mid-term pregnancy) after their separation from the males and their uterine contents were examined for live and dead implantations, the latter comprising dead embryos and deciduas. The main criteria used for the assessment of mutagenicity were the numbers of dead implantations expressed per pregnant female and as a percentage of all implantations.

Numbers of dead implantations, including deciduomas and dead embryos, showed no significant differences among the different groups, thus producing no evidence of any induced post-implantation lethality in mice fed on irradiated diet. Similarly, there was no indication of pre-implantation lethality, since implantation rates remained comparable among different groups. Consumption of irradiated diet did not affect the fertility of mice. Total pre- and post-implantation loss, as indicated by the numbers of live implantations remained comparable among all the groups of mice. The Kolmogorov - Smirnov test (F.O.Massey, The Kolmogorov - Smirnov test for goodness of fit, J.Am.Stat.Ass. 46, 68 (1951) showed overall homogeneity in the mouse population in the different groups in respect of the distribution pattern of dead implantations.

The above-mentioned studies have only limited relevance to the present conflict in the sense all these involve whole diet, whereas the NIN studies are with irradiated wheat. However, the compensatory aspect is that these studies involve a radiation dose of 2.5 Mrad whereas the NIN studies involve a radiation dose of 75 Krad.

At first look the dominant lethal studies of BARC and NIN may appear to have yielded contrary results. The various inadequacies in the experimental designs, statistical analysis as well as interpretations which are biologically unfounded have, unfortunately, raised

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questions regarding the validity and credibility of the NIN data. The experiments of BARC have the benefit of careful designs, appropriate statistical analysis and their data have been interpreted on a sound scientific basis. There is no doubt that BARC has perhaps the advantage of expertise, knowledge of the current literature and above all an impressive animal house. Hence, this Committee after an in-depth study reaches the conclusion that BARC data as well as the NIN data do not demonstrate any mutagenicity of irradiated wheat.




Table 1. Slides previously examined by VL in 1973 and re-examined in 1976 by us

Animal No.	Slide No.	Per cent frequency estimated by VL on 100 cells/slide		Per cent frequency estimated by us on complete enumeration	
R*17/73	VL-2	1/100	1.0	10/5592	0.18
	VL-4	2/100	2.0	7/3420	0.2
28/73	VL-2	0/100	0	2/1935	0.1
	VL-3	0/100	0	3/4108	0.07
	VL-4	0/100	0	4/4452	0.09
	VL-5	0/100	0	2/1812	0.11
31/73	VL-1	0/100	0	3/2214	0.14
	VL-2	0/100	0	2/1727	0.12
	VL-3	0/100	0	6/3715	0.16
	VL-4	0/100	0	5/4891	0.10
35/73	VL-2	0/100	0	1/1646	0.06
	VL-3	0/100	0	2/862	0.23
	VL-4	0/100	0	2/956	0.21
	VL-5	0/100	0	1/832	0.12
37/73	VL-4	0/100	0	6/4146	0.14
	VL-2	0/100	0	3/2925	0.07
39/73	VL-2	0/100	0	2/2746	0.07
	VL-3	0/100	0	3/4092	0.07
	VL-4	0/100	0	4/5016	0.08
	VL-5	0/100	0	2/3732	0.05
*65/73	VL-1	0/100	0	9/10327	0.09
	VL-2	1/100	1.0	8/9686	0.08
	VL-4	1/100	1.0	12/7218	0.17
	VL-5	1/100	1.0	12/4760	0.25
Mean	irradiated wheat		1.0		0.14
Value	Unirradiated wheat		0.0		0.10

*Animals fed on freshly irradiated wheat

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Table 2. Frequency distribution of random starts on Y-axis of the slides observed during test on 24.6.1976.

Class Interval	Frequency
26 - 30	2
31 - 35	5
36 - 40	3
41 - 45	3
45	0
TOTAL	13

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Table 3. Comparison of slides used for tests on 24.6.76 with the corresponding observations made by V.L. in 1973.

Animal No.	Slide No.	Polyploids scored in 1973 Sample Size (100)	Polyploids scored on 24.6.76	
			Sample Size (100)	Sample Size (200)
44/73	2	0	0	0
15/73	2	1	0	0
15/73	3	2	0	0

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Table 4. The number of the scorable cell at which a polyploid was observed in the complete count on animal 17/73

	Slide No. VL-2	VL-4
	342	335
	565	1072
	1167	1163
	1467	1361
	1511	1660
	1615	1665
	1626	2318
	3372	
	4715	
	5043	
Total cells scored	5592	3420

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Table 5. Comparison of frequency estimated by VL based on 100 cells/slide with the frequency estimated by us on complete enumeration

Animal No.	Slide No.	Percent frequency of polyploids on complete enumeration		Percent frequency obtained by VL (100 cells/slide)
302	4	8/4412	0.18	0
303	1	9/7455	0.12	0
304	1	15/7345	0.20	0
	2	15/6335	0.24	0

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SUMMARY OF THE TECHNICAL REPORT ON "THE DATA OF NIN,
HYDERABAD AND BARC, BOMBAY ON THE BIOLOGICAL EFFECTS
OF FRESHLY IRRADIATED WHEAT".

In accordance with the terms of reference in letter No. F.17-45/PH(R&N) of Bharat Sarkar, Ministry of Health and Family Planning, dated October 9, 1975, we, Prof. P.V. SUKHATME of the Maharashtra Association for Cultivation of Sciences, Poona and Dr. P.C. KESAVAN of the School of Life Sciences of the Jawaharlal Nehru University, New Delhi, have critically examined the detailed techniques of the experiments, the appropriateness of the design of the experiments as well as the data collected and the interpretations thereof. This Committee visited the NIN, Hyderabad and also BARC, Bombay twice in order to study the raw data, look at the animal house and seek clarifications on various important questions.

The report is based on the data made available to us by the Ministry with the letter quoted above, the data published by the NIN and BARC in various national and international journals and Annual Reports as well as our analyses of some of the slides prepared by the NIN and BARC. The highlights of our findings are presented below :

1. Effects on lymphocytes in vitro from malnourished children fed freshly irradiated wheat :

In their studies, the NIN had observed that there were no polyploid cells in malnourished children fed

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unirradiated wheat whereas the frequency of incidence of polyploid cells was 1.8% when these children had been fed irradiated wheat. Critical survey of the literature by this Committee revealed that this frequency of 1.8% polyploid cells is well within the normal range of occurrence in healthy human beings. The examination of the raw data of the NIN by this Committee also revealed that the abnormally low frequency of 0.0% of polyploid cells in malnourished children before their exposure to irradiated wheat came from only 20 to 30 cells per child since about 75% of the cells in these malnourished children had chromosomes which exhibited 'fuzzy' appearance and therefore, were not fit for counting. One of the two authors agreed with the doubt expressed by this Committee as to the suitability of these cells for detailed numerical analyses of the chromosomes. The NIN workers also had reported that the number of polyploid and abnormal cells decreased considerably at the end of 16 weeks and by the 24th week their frequency came to normal levels following the withdrawal of these children from irradiated wheat diet. From a biological point of view this observation is conflicting with the fact that the thymic lymphocytes which alone respond to phytohaemagglutinin (PHA) continue to exist in circulation for a time span of the order of years. The Committee, therefore, finds it difficult to agree with the conclusions drawn by the NIN.

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2. Effects on the cytology of bone marrow of rats and mice :

From their studies, NIN observed an increase in the per cent frequency of polyploids in animals fed freshly irradiated wheat. In addition the animals fed with unirradiated wheat gave a value of 0 to 0.03 per cent polyploids in the bone marrow cells.

The Committee examined in detail the data of the earlier experiments of the NIN, the techniques adopted for estimating the frequency of polyploid cells and also the data arising out of joint studies conducted by the NIN and BARC. The Committee also examined a number of slides which had been previously scored by the NIN investigators and also the slides prepared by the NIN and BARC in their joint studies. It was also noted by this Committee that the NIN in their studies in 1973 had obtained low values for polyploid cells in their normal animals and relatively high values in their animals fed irradiated wheat. This Committee on rescoreing some of their slides of control and treated animals did not find that there was any increased incidence of polyploid cells in the bone marrow of animals freshly fed/irradiated wheat. It was felt by this Committee that the contradictory results obtained between this Committee and the NIN on the slides of the NIN could be due to imprecise and inadequate sampling techniques. In order to verify this, the Committee requested the NIN worker to

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rescore the slides on the same basis adopted earlier in their studies. The values obtained from this rescoring by the same worker were nowhere near the figures reported earlier on the same slides from the same animals. The results from these re-analyses revealed that NIN had, unfortunately, underestimated the frequency of polyploid cells in the animals fed unirradiated wheat and overestimated the frequency of polyploid cells in animals fed irradiated wheat.

The statistical analyses of the data from the joint study confirm that the observations made by the NIN technique were indeed subject to large and variable bias so much so that the error variance contributed as much as 70% of the total variance of an observation. The bias arose mainly as a result of faulty selection of the sample in that the central portion of a slide was over-sampled relative to the surrounding area, while the inadequate size of the sample mainly contributed to the large error variance. BARC got over these difficulties by enumerating the slides completely. It is, of course, an expensive and time consuming method, but it is far better to err on the safe side than go about the way the NIN did.

An idea of the size of bias in NIN results can be had from a comparison of their counts with the complete recount made by this Committee. Whereas NIN reported a frequency of 0.6 per cent for polyploids in the treated

series, the recount by complete enumeration gave a figure of only 0.14 per cent. Likewise the recount of the same slides by the NIN using their technique gave a value nearly twice as high as their earlier figure of 0.6 per cent and seven times as high as the value based on complete enumeration thus testifying to the enormous variation. The Committee thus reaches the conclusion that when the NIN results are corrected for bias and error variance, they cannot be considered as inconsistent with those reported by BARC. The differences between NIN and BARC do not therefore lie so much in the adoption of this design or that or this method of analysing variations or that or whether the differences are significant of at 5% or 6% level of significance but in the view of this Committee they mainly lie in the faulty and biased selection of the sample.

This Committee is also convinced, by reference to the literature, that the bone marrow of normal animals of all species contains cells of which a small fraction are polyploids; in rats the frequency is around 0.2 per cent.

3. Effect of freshly irradiated wheat on meiotic chromosomes in mice:

On the basis of their studies, NIN concluded that animals fed on irradiated wheat carried a substantial number of aneuploids on the testis.

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The NIN observation of "aneuploids" in spermatocytes (11% in the control and 22% in the experimental group) of male mice have unfortunately come from accepting slides of a poor quality. This Committee, on examination of a few of these slides, observed that there were large numbers of broken cells with bivalents shattered from the cells. This Committee had occasion to suggest to NIN that if these were not artefacts, the biological consequences would have been that their male mice would have been almost totally sterile, since approximately 44% of the spermatozoa in the control and approximately 88% of spermatozoa in the treated mice would have degenerated. The Committee believes that NIN has accepted these observations as artefacts more so in view of the fact that one of their colleagues associated with this project had suggested the same reasons for the phenomena observed.

4. Effect of irradiated wheat on germ cell survival in male mice and rats :

It is evident both from the publications as well as their discussions with this Committee that the NIN researchers had assumed a very simple model in the formation of spermatocytes whilst the fact is that it is a complex process. Some of the outstanding authorities in this field have defined the spermatogenic cycle in different mammalian species in quantitative terms and these models are widely accepted.

Therefore, the simplistic model advanced by NIN is not reconcilable. Though there could be some disagreement with the ratio of Type A to Type B (which is mainly due to the number of tubules sampled, and the values presented by different workers), the ratio of Type A to RPS (1 : 1) as observed by NIN has not been reported. This ratio (i.e. Type A to RPS) is normally 1:4-6.

In addition there are some more biological inconsistencies which the NIN data revealed. For example, if the NIN data regarding the identification of the tubules and the types of cells thereof are precise and the enumeration of these cells is flawless, there should not have been the sort of data (see Table 2, Page 96 of Int. J. Radiat. Biol. 29, 1976 or see Table 11 page 17 of the NIN Ann. Report 1974) which shows that type B spermatogonia are significantly less in number than type A spermatogonia. This is contrary to the established biological facts since type B, being derived from type A spermatogonia, should at least be equal, if not, more in number.

Above all, the reduced germ cell survival does not reflect on the litter size as per the reported data of the NIN itself. This Committee believes that an over simplified approach to a very intricate and complex process such as the formation of primary spermatocytes (Pl' cells) from the stem cells (A₉) has caused this confusion. In view of such inconsistencies, these data do not fulfil the biological requirements for any analysis.

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5. Dominant lethal studies in rats and mice:

The NIN data on the dominant lethal mutations come from 72 virgin female rats and 108 virgin female mice mated in batches of 2 to 3 per week for 4 weeks to 6 male rats or 9 male mice fed irradiated wheat for 12 weeks. This experimental model would conform to a "Chronic Feeding Study". Since the spermatogenic cycle in rats and mice extends to about 70 days, feeding the animals for 12 weeks on irradiated wheat would affect all the cell stages of spermatogenic cycle. In other words the sperms which contribute to the mating immediately following the feeding schedule would carry the cumulative effects, if any, of the previous 12 weeks of feeding (irrespective of the spermatogenic cycle which is maximally sensitive to the action of the toxic agent). Under these circumstances, the deleterious effects, if any, should reflect as dominant lethal mutations in the mating week I itself. The NIN investigators observed an increase in the DLM during mating weeks III and IV and none in mating weeks I and II. This observation is contrary to what is expected in terms of biological principles.

The NIN was unable to explain the lack of increased DLM in the mating weeks I and II and its occurrence exclusively in mating weeks III and IV. In the earlier stages the NIN had this as a demonstration of sensitivity spectrum, but since this question had been raised, it had refrained from doing so in their publication in International Journal

of Radiation Biology, 1976, 29, No.1, 93-98.

The raw data was analysed by Prof. Sukhatme as early as 1974 and on the basis of those analyses, he had written to the Director, NIN, that, "while the observed F values are not significant at the conventional level of 5%, the probability of its exceeding the observed values is small enough to deserve serious consideration. This probability is of the order of 6 per cent. The differences are seen to increase from week to week with the percentage incidence of lethals twice as large in the fourth week as in the first, both for well-fed and depleted groups". Notwithstanding these observations, the authors had published their results in the journal mentioned above claiming levels of significance at 1 per cent and below.

The Committee has come to the conclusion on the basis of biological expectations that the differences seen from week to week is a demonstration of a very high experimental error and therefore statistical significance at 6 per cent is of no consequence.

Apart from these considerations there is the fundamental question as to where the dead embryos come from, while the NIN data demonstrated that neither the total number of implantations nor the live births between animals fed irradiated or unirradiated wheat have been even marginally affected.

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Such inexplicable biological observations, coupled with the results of statistical reevaluation have lead this Committee to record that the earlier conclusions of NIN, that DLM is increased as a consequence of feeding freshly irradiated wheat are not founded. In arriving at this judgement the Committee had also evaluated the work carried out by BARC using 3 different feeding schedules wherein DLM was not demonstrable in animals fed with freshly irradiated wheat.

GENERAL REMARKS

This Committee finds that the bulk of the NIN data are not only mutually contradictory but also are at variance with the well established facts of biology. The Committee is satisfied that when the NIN data are corrected for biases which have given rise to these contradictions, the results obtained in animals fed freshly irradiated wheat cannot be considered as incompatible with those obtained in animals fed unirradiated wheat.

The Committee could not also observe that malnutrition per se changes the end point effects.

In drawing these conclusions, this Committee would like to reaffirm its strict adherence to the terms of reference (to identify the cause of discrepancy between the data of NIN and BARC) and nothing beyond it.